Biochimica et Biophysica Acta 1817 (2012) 1339–1345

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Deletion of *psbJ* leads to accumulation of Psb27–Psb28 photosystem II complexes in *Thermosynechococcus elongatus*☆

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ARTICLE INFO

Article history:
Received 8 December 2011
Received in revised form 14 February 2012
Accepted 15 February 2012
Available online 22 February 2012

Keywords:
Photosystem II
Cyanobacteria
Psb27
Psb28
PsbJ

ABSTRACT

The life cycle of Photosystem II (PSII) is embedded in a network of proteins that guides the complex through biogenesis, damage and repair. Some of these proteins, such as Psb27 and Psb28, are involved in cofactor assembly for which they are only transiently bound to the preassembled complex. In this work we isolated and analyzed PSII from a Δ*psbJ* mutant of the thermophilic cyanobacterium *Thermosynechococcus elongatus*. From the four different PSII complexes that could be separated the most prominent one revealed a monomeric Psb27–Psb28 PSII complex with greatly diminished oxygen-evolving activity. The MALDI-ToF mass spectrometry analysis of intact low molecular weight subunits (<10 kDa) depicted wild type PSII with the absence of PsbJ. Relative quantification of the PsbA1/PsbA3 ratio by LC-ESI mass spectrometry using ^{15}N labeled PsbA3-specific peptides indicated the complete replacement of PsbA1 by the stress copy PsbA3 in the mutant, even under standard growth conditions (50 μmol photons m^{-2} s^{-1}). This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

Photosystem II (PSII) is a large membrane protein complex of the photosynthetic electron transfer chain in cyanobacteria and chloroplasts of higher plants that catalyzes a unique reaction: the light-driven oxidation of water [1,2]. Remarkable improvements in the X-ray structural analysis of PSII led to a detailed picture of the static complex on the molecular level [3]. In addition to the surrounding protein, in particular the 3D structure of the water-oxidizing cluster (WOC) with four manganese ions, one calcium ion and two close by chloride ions with unknown functions [4] were able to be resolved. Among the 20 permanent cyanobacterial PSII subunits shown in the recent crystal structures [3,5], the core proteins D1 and D2 bind all redox-centers of the intrinsic electron transfer chain, whereas most of the chlorophylls are located inside the intrinsic antennae proteins CP43 and CP47. Three extrinsic proteins (PsbO, PsbU, PsbV) that are attached to the lumenal surface of cyanobacterial PSII protect the WOC and have an impact on its assembly [6,7].

One striking feature of the complex is the 13 low molecular weight (LMW) subunits (<10 kDa) [8]. Among them is the PsbJ subunit with a single transmembrane helix and a molecular mass of 4.1 kDa. Together with the nearby subunits PsbE and PsbF, PsbJ seems to form the entrance for a quinone channel that provides access to the Q_B binding site within PSII [5]. Deletion of *psbJ* in *Synechocystis* sp. PCC 6803 (hereinafter referred to as *Synechocystis* 6803) leads to diminished oxygen evolution rates of PSII [9,10] and a longer lifetime of reduced Q_A [9,11], suggesting that PsbJ is involved in the regulation of forward electron transfer from Q_A to the plastoquinone pool. In higher plants, deletion of *psbJ* [9,11–14] results in loss of photoautotrophic growth, high light sensitivity and defects in the assembly of the extrinsic proteins PsbP and PsbQ.

Interestingly, the effect of *psbJ* deletion in the thermophilic cyanobacterium *Thermosynechococcus elongatus* seems to be dependent on the D1 protein. Cyanobacteria usually encode a small family of different D1 proteins [15,16]. In *T. elongatus* three copies of the *psbA* gene [17] encode D1 proteins: While PsbA1 is expressed under normal light conditions (50 μmol photons m^{-2} s^{-1}), the D1 pool is rapidly

Abbreviations: PSII, Photosystem II; Q_B/Q_A, plastoquinone B/A; Chl, chlorophyll; IMAC, immobilized metal affinity chromatography; IEC, ion exchange chromatography; WOC, water-oxidizing complex; Cyt, cytochrome; WT, wild type; RC47, CP43–less PSII complex; LMW, low molecular weight; PTM, post translational modification; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; BN-PAGE, blue native polyacrylamide gel electrophoresis; MALDI-ToF, matrix assisted laser desorption ionization time-of-flight mass spectroscopy; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry

☆ This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

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doi:10.1016/j.bbabio.2012.02.017

exchanged under high light conditions (500 μmol photons m^{-2} s^{-1}). This occurs both on the transcript and protein level by expression of the alternative copy PsbA3, resulting in functional differences in the mature complex [18,19]. In contrast, the second alternative copy PsbA2 seems to be induced under microaerobic conditions [20]. Combination of the *psbJ* deletion with a strain expressing only PsbA1 or PsbA3, respectively, leads to interesting differences [21]: while isolated PSII from the Δ*psbJ*/PsbA3 mutant seems to be structurally unaffected, PSII complexes from the Δ*psbJ*/PsbA1 mutant show a reduced stability of the dimer and a lower oxygen-evolving activity. Moreover, many of the low molecular weight subunits are lacking in this preparation.

In addition to the 20 static subunits of PSII, there is an increasing network of proteins that are involved in biogenesis, repair and adaptation of this complex. These proteins play only a transient role in the PSII life cycle [6,17,22], with Psb27 being one of the most studied transient PSII factors. Psb27 was found in several PSII preparations [23–26] and seems to be involved in the assembly of the WOC during biogenesis and repair [27–32]. Although the structure of Psb27 has been resolved and models for its binding to PSII are available [33–39], its precise role within the PSII life cycle remains unclear. Psb27 was also shown to be involved in the repair cycle after deactivation of PSII [40], in PSII supercomplex remodeling in plants [41] and in association with PSI [38].

Psb28 is a small (~13 kDa) soluble protein. It was identified in PSII complexes isolated from cyanobacteria [23,24] and red algae such as *Galdieria sulphuria* [42]. In *Arabidopsis thaliana* it is encoded by a nuclear gene (AT4g28660) and it seems to have some partial sequence similarity with PsbW [43], which is only present in higher plants. But in contrast to Psb28, PsbW is smaller (~7 kDa) and it is anchored in the thylakoid membrane by a single transmembrane helix. The solution structure of *Synechocystis* 6803 Psb28 was recently solved by NMR spectroscopy [44]. Besides being predominantly associated with the CP43–less PSII subcomplex (RC47) and, in small amounts, with free CP47 in *Synechocystis* 6803 [45], the authors suggested that Psb28 might be involved in the regulation of chlorophyll availability during PSII (and PSI) biogenesis, although its precise role still has to be elucidated. Interestingly, the *pgsA* deletion mutant of *Synechocystis* 6803 that is defective in the biosynthesis of phosphatidylglycerol shows elevated levels of Psb28 associated with PSII monomers and to a lesser extent with PSII dimers [46].

In this study we isolated and characterized PSII complexes from a *psbJ* deletion mutant of *T. elongatus*, resulting in the accumulation of monomeric PSII complexes with both Psb27 and Psb28. While all other small PSII subunits are present, with the exception of PsbJ and PsbY, PsbA1 is completely replaced by the D1 stress copy PsbA3 in the isolated particles.

2. Materials and methods

2.1. Construction of the psbJ deletion mutant

The *psbJ* gene (tsr1544) and the flanking up and downstream sequences from *T. elongatus* were amplified by use of the oligonucleotide primer pair TEpsbJ_for (5′-CTTGGTTCTTGCGTTGCTCT-3′) and TEpsbJ_rev (5′-TTGGACTCCTCTGGCTACAA-3′) and cloned into the vector pUC19 (Fermentas). For deactivation of the gene, a kanamycin resistance cartridge was introduced via an NgoMIV restriction site. Cells of a CP43-His strain of *T. elongatus* were transformed by electroporation [47] with this vector to produce the mutant strain. Mutant cells were selected by gradually adding kanamycin to the medium up to a concentration of 40 μg/ml. Single colonies were obtained by plating the mutant strain onto selective agar (40 μg/ml kanamycin). Segregation was checked via PCR using the TEpsbJfor and TEpsbJrev primers.

2.2. Culture conditions

T. elongatus cultures were routinely grown in liquid BG11 medium at 45 °C and bubbled with CO_2-enriched (5%) air. The growth light intensity was 50 μmol photons m^{-2} s^{-1} in all experiments. For isotopic labeling of all proteins, the respective culture was grown on a modified BG11 medium, which contained $^{15}NH_4Cl$ as the sole nitrogen source.

2.3. Isolation and PAGE analysis of His-tagged PSII

Purification of His-tagged PSII by nickel affinity chromatography followed by anion exchange chromatography and oxygen-evolving activity measurements of isolated PSII were performed according to Nowacyzk et al. [28]. PSII complexes were analyzed by BN-PAGE and SDS-PAGE according to Grasse et al. [40].

2.4. Mass spectrometry analysis

Intact PSII subunits were analyzed by MALDI-ToF mass spectrometry according to El-Mohsnawy et al. [48]. Preparation of samples with tryptic peptides and mass spectrometry analysis was carried out according to Nowaczyk et al. [49], and PsbA1/PsbA3 peptides were quantified according to Sander et al. [18].

3. Results and discussion

3.1. A novel monomeric Psb27–Psb28 PSII complex is the major PSII species isolated from ΔpsbJ mutant cells

The *psbJ* gene (tsr1544) was disrupted by a kanamycin-resistance cassette, which was introduced into the gene via the NgoMIV restriction site (Fig. 1a). Complete segregation of the mutant allele in the Δ*psbJ* strain was confirmed by PCR (Fig. 1b), with the 250 base pair signal corresponding to wild type (WT) and the 1700 base pair signal to the Δ*psbJ* sequence, respectively. Δ*psbJ* mutant cells, which were grown under standard conditions (50 μmol photons m^{-2} s^{-1} and 45 °C), showed no significant differences in growth rate and oxygen-evolving activity in comparison with WT cells (data not shown).

PSII complexes have been isolated by metal affinity chromatography via the CP43-His subunit, followed by ion exchange chromatography (IEC). The elution profile of IEC (Fig. 2A) shows four peaks corresponding to four different PSII species: The most prominent, Peak I, represents a monomeric PSII fraction (Fig. 2B) with very low oxygen-evolving activity (237 ± 109 μmol O_2 mg^{-1} Chl h^{-1}). Peak II consists mainly of dimeric PSII, but also contains some PSII monomers (Fig. 2B), probably due to some overlap with peak I. Although this fraction exhibits oxygen evolution activity (662 ± 145 μmol O_2 mg^{-1} Chl h^{-1}), this is much lower compared to active PSII complexes isolated from WT cells with activities ranging from 3000 to 5000 μmol O_2 mg^{-1} Chl h^{-1} [40]. Peaks III and IV (Fig. 2A) correspond to dimeric PSII with oxygen evolution rates of 620 ± 87 μmol O_2 mg^{-1} Chl h^{-1} and 137 ± 75 μmol O_2 mg^{-1} Chl h^{-1}, respectively. Fig. 2C shows a SDS-PAGE analysis of the different PSII complexes. While fractions I, II and III seem to contain the PsbO subunit at stoichiometric amounts, fraction IV shows only traces of this subunit. Moreover, the extrinsic proteins PsbV and PsbU could not be detected in any of the fractions. Psb27 and Psb28 were identified in peak I and also in peak IV, albeit in substoichiometric amounts. These results are summarized in Table 1.

The most striking differences between the PSII preparations from WT [28,40] and Δ*psbJ* mutant cells are the presence of Psb28, which was not previously observed, as well as the reduced oxygen evolving activity (~10%) in preparations of the mutant. This stands in contrast to the unaltered activity of the whole cells and may be due to a limited stability of the isolated complex. Routinely, the WT preparation

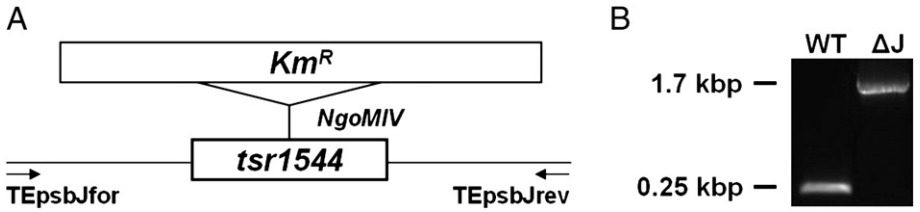

Fig. 1. Generation of the Δ*psbJ* mutant strain. *A*, the Δ*psbJ* mutant strain was generated by introducing a kanamycin resistance cassette into the *psbJ* gene (tsr1544) by use of the restriction enzyme NgoMIV. *B*, segregation of the Δ*psbJ* mutant strain was checked by PCR with the gene-specific primer pair TEpsbJfor and TEpsbJrev.

contains two clearly separated monomeric PSII fractions, of which one is lacking the three extrinsic proteins PsbO, -V and -U. Instead, this fraction contains Psb27 and shows almost no oxygen-evolving activity. The second monomeric PSII fraction contains the three extrinsic proteins and is highly active while lacking Psb27 [28]. Apparently, these two fractions could not be separated in the Δ*psbJ* PSII preparation, with fraction I showing both PsbO and Psb27 and an activity amounting to about 35% of the most active fraction. For this reason, fraction II might correspond to active dimeric PSII, fraction III to dimeric PSII with reduced activity and fraction IV to inactive, dimeric PSII of the WT PSII preparation [40]. However, this assignment is still ambiguous. In contrast to the WT preparation, fraction IV shows some reduced activity and it seems to contain only substoichiometric amounts of Psb27 (and Psb28). Possibly this fraction represents a mixture of two or more PSII fractions which could not be separated.

3.2. MALDI-ToF analysis of intact LMW-PSII subunits reveals no major differences between PSII isolated from WT and ΔpsbJ mutant cells

In addition to SDS-PAGE analysis (Fig. 2C), the protein composition of purified PSII complexes was analyzed by MALDI-ToF mass spectrometry (Fig. 3). Although the resolution of this technique is rather limited compared to other mass spectrometry methods such as LC-ESI-MS/MS [50], it allows the detection of all 13 LMW subunits in isolated PSII complexes (Table 2), which are usually only poorly resolved by SDS-PAGE. Fig. 3A shows that even PsbJ and PsbM could be clearly distinguished in WT samples, although their molecular mass differs only by 7 Da. Additionally, PsbJ seems to be present in an oxidized form (+16 Da) which might be artificially induced by sample preparation. Both PsbJ peaks disappear in samples isolated from the Δ*psbJ* mutant (Fig. 3A). Mass spectrometry analysis of intact proteins also yields information on post-translational modifications [51], although the precise assignment of a certain modification is dependent on the accuracy of the mass determination, especially if no fragmentation data are available. Table 2 lists some examples for post translational modifications (PTMs) which provide feasible explanations for the determined masses. However, they still have to be confirmed by other methods such as top-down mass spectrometry analysis [42]. Based on the MALDI-ToF data, most of the small PSII subunits still retain their formulated N-terminal methionine residue, which is also in agreement with data derived from eukaryotic PSII samples [42,52]. This is a common modification caused by the initiation of translation and limited to the N-terminal methionine [53]. Usually, the formyl group is removed by a deformylase [54] followed by cleavage of the whole methionine residue, provided there is an amino acid

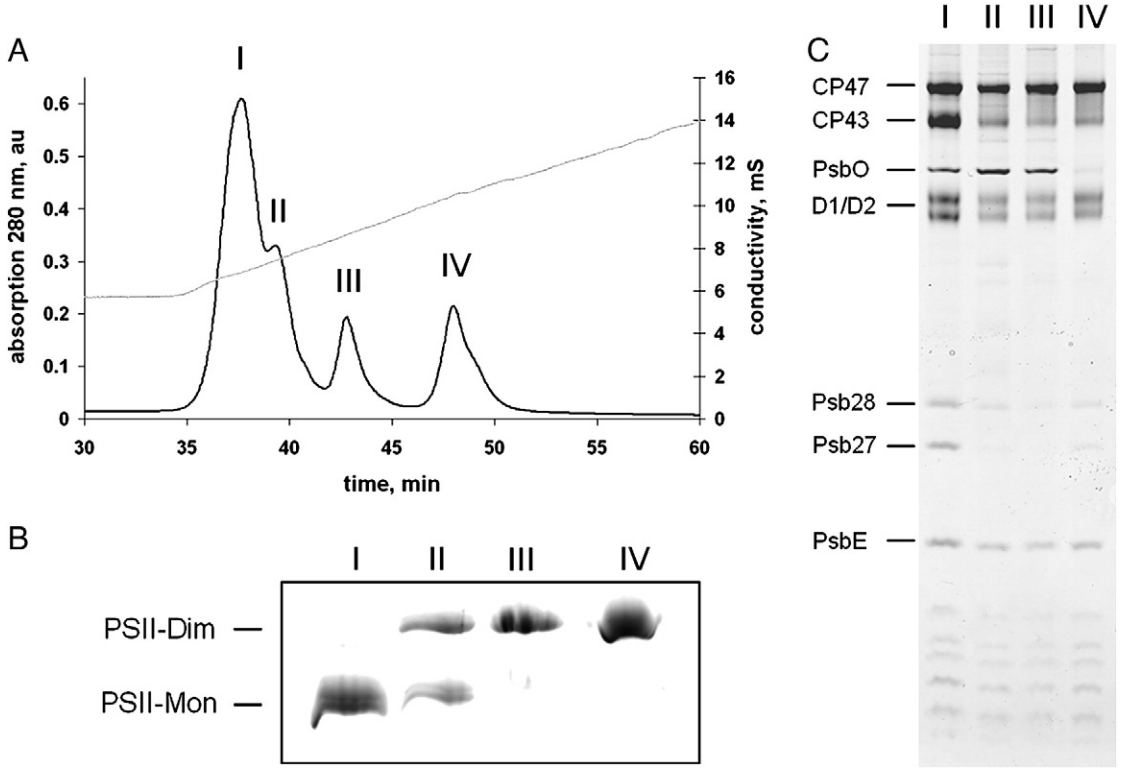

Fig. 2. Isolation and characterization of PSII from *T. elongatus* Δ*psbJ*/CP43-His. A, elution profile (280 nm) of ion exchange chromatography (IEC) after immobilized metal affinity chromatography (IMAC) shows four different peaks. au, absorbance units; mS, millisiemens. The corresponding PSII complexes (1 μg Chl) were analyzed by BN-PAGE (B) and SDS-PAGE (C).

Table 1
Characterization of PSII complexes isolated from Δ*psbJ* cells ($n=3$).

Δ*psbJ*-PSII	I	II	III	IV
Monomer/Dimer	Monomer	Monomer/Dimer	Dimer	Dimer
Activity ($\mu mol \times mg^{-1}$ Chl $\times h^{-1}$)	237 ± 109	662 ± 145	620 ± 87	137 ± 75
Lumenal proteins	Psb27, PsbO	PsbO	PsbO	(Psb27)
Cytoplasmic proteins	Psb28	–	–	(Psb28)

Table 2
MALDI-ToF analysis of intact PSII subunits.

Subunit	Modification	$Mass_{meas}$	$Mass_{calc}$	Δ
PsbE	NH_2-A^2	9442	9443	−1
PsbH	NH_2-A^2	7223	7224	−1
PsbZ	fM	6792	6793	−1
Psb30	fM	5066	5066	0
PsbF	Ac-T^2	4977	4977	0
PsbY	NH_2-(f)M^3	4614	4614	0
PsbI	fM	4434	4434	0
PsbL	–	4297	4298	−1
PsbX	NH_2-T^{11}	4188	4189	−1
PsbK	NH_2-K^{10}	4100	4100	0
PsbJ	NH_2-(f)M^2	4002	4002	0
PsbM	fM	4010	4009	1
PsbT	fM	3903	3904	−1

All masses are average masses (Da). fM, N-terminal formyl methionine; Ac, acetylation; index number, N-terminal amino acid.

with a small side chain (such as alanine) in the second position [55]. According to the crystal structure of PSII [3,5], the N-termini of all PSII subunits with an N-terminal formyl-methionine seem to orientate themselves towards the lumenal space [5,56]. With the exception of PsbZ, all of them carry a large amino acid at position +2.

Considering the determined masses, the identity of the first amino acid should be different in some cases from the sequence available in Cyanobase (http://genome.kazusa.or.jp/cyanobase). This is indicated in Table 2 for PsbJ and PsbY by NH_2-(f)M^2 and NH_2-(f)M^3, respectively. Additionally, the sequence comparison for PsbX (data not shown) indicates that NH_2-(f)M^{10} might be the real N-terminus of the *T. elongatus* sequence, although it is known for PsbK that this subunit is N-terminally processed [57]. In this study PsbF seems to be the only PSII subunit that is acetylated, most probably at the N-terminus.

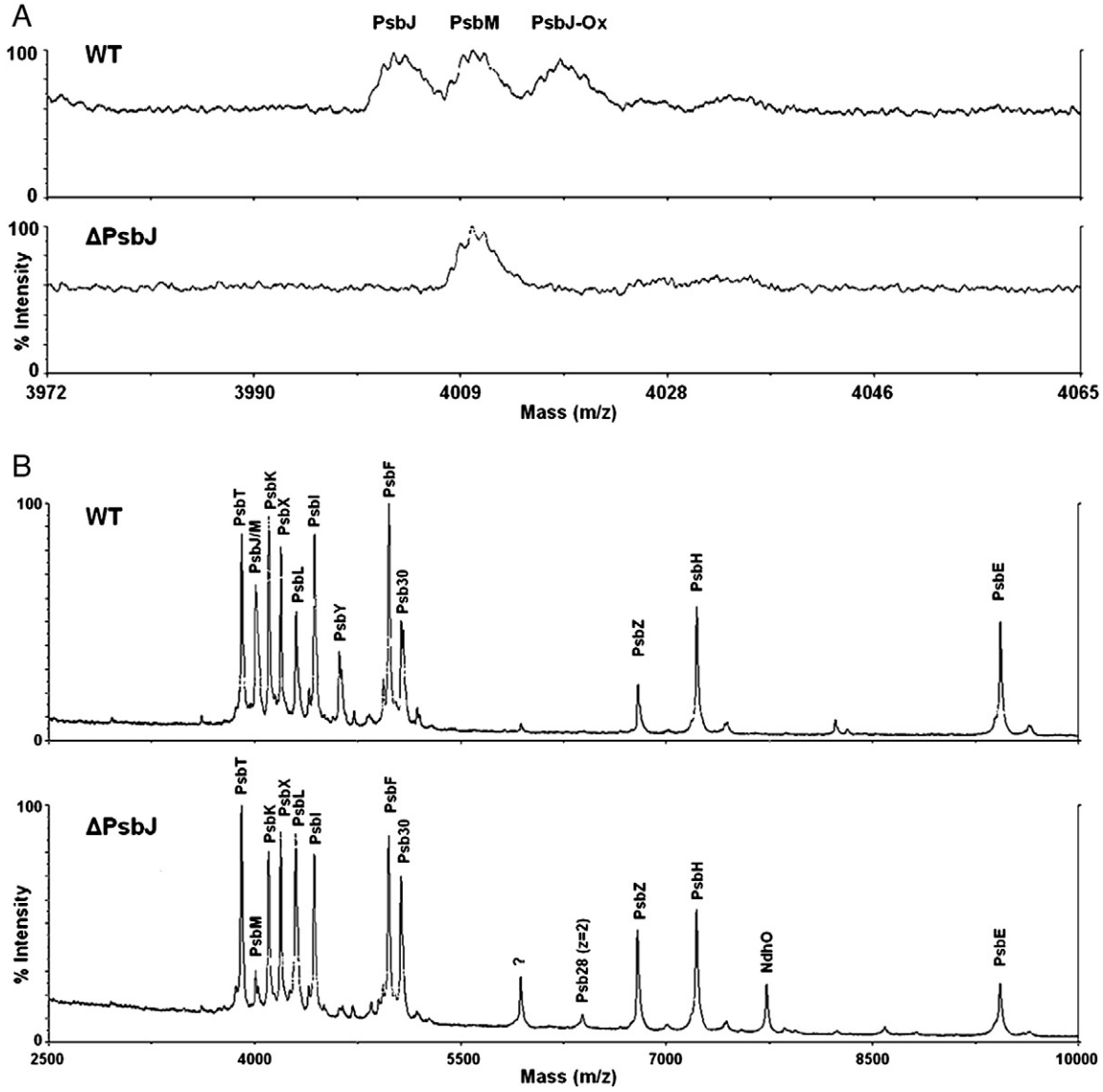

Fig. 3. MALDI-ToF mass spectrometry analysis of intact PSII subunits. PSII complexes isolated from WT (CP43-His) or Δ*psbJ* mutant cells were mixed with sapinic acid matrix solution and directly applied for MALDI-ToF analysis. A, mass range around fM^2-PsbJ (4002 Da), fM-PsbM (4010 Da) and fM^2-PsbJ-Ox (4018 Da). All masses are given as average masses (Da). B, mass range between 2500 Da and 10,000 Da for the analysis of all 13 low molecular weight subunits of PSII (see Table 2).

In *Galdieria sulphuria* PsbF, PsbJ and PsbL are acetylated [42] whereas in *Hordeum vulgare* PsbF, PsbJ and PsbTc show this type of modification [58]. In contrast to eukaryotic proteins, where N-terminal acetylation is a common post-translational modification [59], this is rather unusual in bacteria like *E. coli* [60], but as it is the functional role of this type of PTM remains unclear in all cases.

Altogether, comparison of MALDI-ToF mass spectrometry data from WT and mutant PSII (Fig. 3) reveals no major differences in LMW protein composition, except for the lack of PsbJ. PsbY might be missing in the mutant, but this protein is easily lost during preparation even in WT samples (data not shown). It is also missing in the most recent crystal structure of PSII [3]. Additionally, only the spectrum of PSII from the Δ*psbJ* mutant (Peak I, Fig. 3B) shows an unassigned peak at 5939 Da, the peak for double charged Psb28 (6394 Da) and a contamination of NdhO [49]. Subunits of the NDH-1 complex are common contaminations of PSII samples, which have been purified by IMAC, as NdhF1 of *T. elongatus* contains a histidine-rich region, which readily binds to the metal affinity column [61].

3.3. Relative quantification of PsbA1 and PsbA3 shows that the D1 pool is completely changed to PsbA3 in the ΔpsbJ mutant

According to a recent report [21] ΔPsbJ–PsbA1–PSII complexes show several structural differences from ΔPsbJ–PsbA3–PSII. Compared with WT PSII, ΔPsbJ–PsbA3–PSII isolated by one-step metal affinity chromatography exhibits a similar amount of dimers, a similar oxygen evolution rate and an identical subunit composition. In contrast, ΔPsbJ–PsbA1–PSII dimers are much more unstable, the oxygen-evolution rate is lower and many of the small subunits are missing. We therefore quantified the relative amount of PsbA1 and PsbA3 in our ΔPsbJ-PSII preparation by mass spectrometry. For this, PSII samples, which had been isolated from the Δ*psbJ* mutant grown on a ^{14}N nitrogen source, were mixed with PsbA3–PSII complexes which had been isolated from a Δ*psbA1*/Δ*psbA2* mutant [18] grown on a ^{15}N-enriched media. The mix was applied to SDS-PAGE and proteins corresponding to the PsbA band were digested by trypsin followed by mass spectrometry analysis (Fig. 4). The PsbA1/PsbA3 ratio in WT (Fig. 4C) and Δ*psbJ* (Fig. 4A)

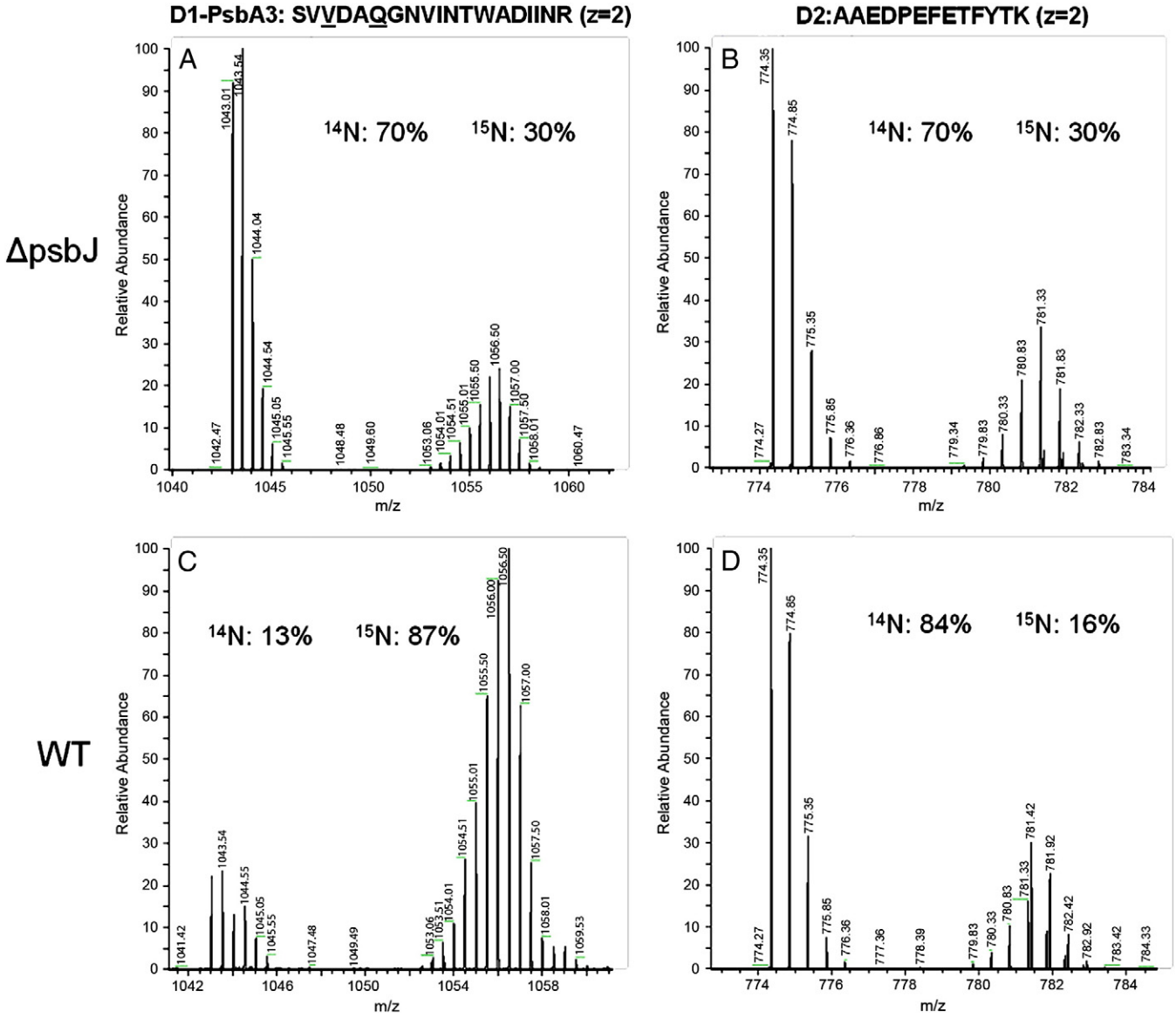

Fig. 4. Relative quantification of PsbA1 and PsbA3 in isolated PSII complexes. PSII complexes (Peak I) isolated from Δ*psbJ* and WT cells were mixed in a 1:1 ratio based on chlorophyll concentration with ^{15}N enriched PSII complexes isolated from ΔpsbA1/ΔpsbA2 cells and applied for SDS-PAGE analysis. After excising, the D1-PsbA band proteins were digested with trypsin followed by LC-ESI-MS/MS analysis. The relative amount of ^{14}N and ^{15}N peptides was determined for the D1-PsbA3 peptide SVVDAQGNVINTWADIINR and the D2 peptide AAEDPEFETFYTK in WT and Δ*psbJ*-PSII complexes. z, charge of peptides.

samples was calculated based on the $^{14}N/^{15}N$ ratio of PsbA3 specific peptides by the following equation: $(A/B \times C - D)/D$; *A*: $\%^{14}$N-D2; *B*: $\%^{15}$N-D2; *C*: $\%^{15}$N-PsbA3; *D*: $\%^{14}$N-PsbA3

The $^{14}N/^{15}N$ ratio of the D2 peptide (Fig. 4B and D) was used to correct the mixing ratio of the samples. Surprisingly, our results clearly show that the PsbA pool is completely changed to PsbA3 (100%) in the mutant (Fig. 4A), while in WT PSII PsbA1 is always the major component (97%) under the tested conditions (Fig. 4B). These results were confirmed by analysis of the PsbA3 peptides EWELSYR and ATAVFLYPIGQGSF (data not shown).

4. Conclusion

Although the oxygen-evolving activity of whole cells is not affected in the *ΔpsbJ* strain, isolated PSII complexes reveal several structural and functional differences from WT: The major PSII fraction is a monomeric complex with strongly reduced activity that contains two additional proteins Psb27 and Psb28, which are involved in the biogenesis and repair of PSII. Usually, Psb27 is bound to inactive PSII [28,40], but it might be also associated with active complexes in *Synechocystis* 6803 [26]. Psb28 was shown to be mainly associated with free CP47 and the RC47 subcomplex in *Synechocystis* 6803, but it was reported to be present only in low amounts in monomeric PSII and missing in dimeric PSII fractions [45]. Moreover, the authors observed in a Psb28 deletion strain a huge accumulation of the chlorophyll precursor protoporphyrin IX, while the amount of RC47 was reduced and free CP47 could not be detected. In conclusion, they suggested that Psb28 plays a role in chlorophyll integration into CP47/RC47 [45]. The present study shows Psb28 predominantly binds to monomeric PSII and—in substoichiometric amounts—to a dimeric PSII fraction isolated from the Δ*psbJ* mutant. Presently, we cannot rule out the possibility that this dimeric complex represents the major fraction in the thylakoid membrane, which is monomerized artificially during the isolation procedure. However, the fact that this PSII fraction contains both CP43 and CP47 indicates an additional role of Psb28 after assembly of the core complex or in the PSII repair cycle—at least in the Δ*psbJ* mutant. The reduced stability of the isolated ΔPsbJ-PSII complexes compared to WT may explain their strongly reduced oxygen-evolving activity: Either ΔPsbJ-PSII is easily damaged during the multi-step isolation procedure, or these complexes are rapidly deactivated during the activity measurements.

Most remarkably, we could observe the exchange of *psbA1* by the (light) stress copy *psbA3* in the Δ*psbJ* mutant under standard growth light conditions (50 μmol photons m^{-2} s^{-1} and 45 °C). As in WT, the expression of *psbA3* was shown to be induced by high light conditions (500 μmol photons m^{-2} s^{-1}, [18]). This report is the first indication that—independent of the light quantity—PsbA3 could play a more general role in the stress response of *T. elongatus,* but this has to be verified by further experiments.

Acknowledgements

We appreciate the support in the form of grants from the Deutsche Forschungsgemeinschaft (SFB 480, Project C1; M.M.N. & M.R.) and the Protein Research Department of the Ruhr-Universität Bochum (M.M.N. & M.R.). We would also like to thank Melanie Völkel, Regina Oworah-Nkruma and Claudia König for their excellent technical support.

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